Attorney's Docket No. 41565/192844

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: James Francis GLOVER et al.
Appl No.: [Not yet assigned] Group Art Unit: [Not yet assigned]
Filed: December 22, 1999
For: ANGIOTENSIN DERIVATIVES

December 23, 1999

Assistant Commissioner for Patents
BOX PATENT APPLICATION
Washington, DC 20231

TRANSMITTAL LETTER FOR
NEW CONTINUATION APPLICATION

Dear Sir:

This is a request for filing the attached new patent application as a continuation application under 37 CFR 153(b) of the U.S. designation of International Application No. PCT/GB98/01833 (published as WO98/58952), entitled ANGIOTENSIN DERIVATIVES and filed on 23 June 1998 claiming priority of Great Britain Patent Application Nos. 9713361.5 (filed 24 June 1997) and 9808696.0 (filed 23 April 1998) by the following named inventors:

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Please direct all correspondence and telephone calls in this application to:

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601 Pennsylvania Avenue, N.W.
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Prior to calculation of the filing fee, please enter the enclosed preliminary amendment. This amendment is submitted to eliminate multiple dependencies in the claims. Check No. 2204 in the amount of $380.00 in payment of the filing fee for a small entity is enclosed at this time. Also enclosed is the executed Statement Claiming Small Entity Status. The Commissioner is hereby authorized to credit overpayments or charge to Deposit Account No. 16-0605 any fees required under 37 CFR 1.16 (National filing fees) and/or under 37 CFR 1.17 (National application processing fees).

An executed assignment is also submitted herewith for recordal with the appropriate recordal cover sheet and Check No. 2203 in the amount of $40.00 in payment of the assignment recordal fee.

Priority of Great Britain Patent Application Nos. 9713361.5 (filed 24 June 1997) and 9808696.0 (filed 23 April 1998) is claimed under 35 USC 119.

Respectfully submitted,

Karen Lee Orzechowski
Registration No. 31,621

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Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Dear Sir:

Prior to calculation of the filing fee, please amend the above-identified application as follows:

In The Claims:

Please amend claims 3-5, 7-18, 20___ as follows:

3. (Amended) The use as claimed in claim 1 [or claim 2] wherein the carrier binding moiety contains an amino acid residue having a reactive side chain.

4. (Amended) The use as claimed in [any one of the preceding claims] claim 3 wherein the carrier binding moiety is a peptide extension at the N- or the C-terminus of an angiotensin peptide moiety.

5. (Amended) The use as claimed in [any one of the preceding claims] claim 1 wherein the angiotensin derivative is of Formula I

$$((A)\text{-}X_n)_m\text{-}L_p\text{-}Y\text{-}[L_q(X_r\text{-}(A))_s]_t \qquad (I)$$

wherein

A represents an angiotensin peptide moiety;

X represents an amino acid;

Y represents an amino acid having a side chain with a free –SH, -OH or –COOH group;

L represents an organic linker capable of binding a group ((A)-X_n) – at one or more sites, e.g. capable of binding up to 10 (A)X_n moieties;

n and r are each = 0-20;

m and s are each ≥1, e.g. 1 to 10, preferably 1, 2, 3 or 4; and

p, q and t are each 0 or 1;

wherein X may be attached at the N- or C-terminus of the angiotension peptide moiety with the proviso that if m≥2, then p=1, or if s≥2, then q=1.

7. (Amended) The use as claimed in [any one of claims 5 or 6] claim 5 wherein L is a peptide chain.

8. (Amended) The use as claimed in [any one of claims 5 to 7] claim 5 wherein n and r are each 0-10.

9. (Amended) The use as claimed in [any one of claim 5 to 8] claim 5 wherein m and s are each <8.

10. (Amended) The use as claimed in [any one of claims 5 to 9] claim 5 wherein X is an amino acid having no side chain or a hydrocarbyl side chain (preferably an alkyl, C_{3-7} cycloalkyl or cycloalkenyl, C_{3-7} cycloalkyl- or cycloalkenyl-alkyl, alkaryl, aralkyl or alkarylalkyl moiety in which each alkyl moiety may be saturated or unsaturated and contains up to 6 carbons and each aryl moiety is preferably a phenyl ring), particularly preferably an aliphatic side chain.

11. (Amended) The use as claimed in [any one of claims 5 to 10] claim 5 wherein X

is glycine, alanine, β-alanine, valine, leucine or isoleucine.

12. (Amended) The use as claimed in [any one of claims 5 to 11] claim 5 wherein the angiotensin derivative is selected from

$$(A)\text{-}X_n\text{-}Y \qquad (II)$$

$$(A)\text{-}X_n\text{-}L\text{-}Y \qquad (III)$$

$$((A)\text{-}X_n)_m\text{-}L\text{-}Y \qquad (IV)$$

$$(A)\text{-}X_n\text{-}L\text{-}Y\text{-}L\text{-}X_r\text{-}(A) \qquad (V)$$

wherein A, X, L, n and r are as hereinbefore defined and $m \geq 2$.

13. (Amended) The use as claimed in [any one of the preceding claims] claim 1 wherein the angiotensin derivative is selected from

(A)-Gly Cys

(A)-Cys

(A)-Tyr

N-acetyl-Cys-(A)

Tyr - (A)

N-acetyl-Cys-Gly-(A)

Cys - (A)

(A) - N-acetyl-Cys

where A is angiotensin I or II.

14. (Amended) The use as claimed in [any one of the preceding claims] claim 1 wherein the angiotensin derivative elicits a cross-reactive immune response with angiotensin I,

angiotensin II, and/or angiotensinogen molecules.

15. (Amended) The use as claimed in [any one of the preceding claims] Claim 1 wherein the angiotensin derivative is conjugated to a carrier.

18. (Amended) The use as claimed in [any one of the preceding claims] claim 1 wherein said disease is congestive heart failure or hypertension.

20. (Amended) A pharmaceutical composition comprising an angiotensin derivative as defined in claim 5 [any one of claims 1-14, or a conjugated angiotensin derivative as defined in any of claims 15 to 17,] together with one or more pharmaceutically acceptable carriers or excipients.

21. (Amended) An angiotensin derivative as defined in [any one of claims 1-17] claim 5 for use in therapy.

23. (Amended) An angiotensin derivative as claimed in [any one of] claim 22 wherein L is a peptide chain.

24. (Amended) An angiotensin derivative as claimed in [any one of claims 22 or 23] claim 22 wherein n and r are each 0-10.

25. (Amended) An angiotensin derivative as claimed in [any one of claims 22 to 24] claim 22 wherein m and s are each ≤ 8.

26. (Amended) An angiotensin derivative as claimed in [any one of claims 22 to 25] claim 22 wherein X is an amino acid having no side chain or a hydrocarbyl side chain (preferably an alkyl, C_{3-7} cycloalkyl or cycloalkenyl, C_{3-7} cycloalkyl-or cycloalkenyl-alkyl, alkaryl, aralkyl or alkarylalkyl moiety in which each alkyl moiety may be saturated or unsaturated and contains up to

6 carbons and each aryl moiety is preferably a phenyl ring), particularly preferably an aliphatic side chain.

27. (Amended) An angiotensin as claimed in [any one of claims 22 to 26] claim 22 wherein X is glycine and alanine, β-alanine, valine, leucine or isoleucine.

28. (Amended) An angiotensin derivative as claimed in [any one of claims 22 to 27] claim 22 selected from

$$(A)\text{-}X_n\text{-}Y \qquad (II)$$

$$(A)\text{-}X_n\text{-}L\text{-}Y \qquad (III)$$

$$((A)\text{-}X_n)_m\text{-}L\text{-}Y \qquad (IV)$$

$$(A)\text{-}X_n\text{-}L\text{-}Y\_L\_S_r\text{-}(A) \qquad (V)$$

wherein A, X. L, n and r are as hereinbefore defined and $m \geq 2$.

29. (Amended) An angiotensin derivative as claimed in [any one of claims 22 to 28] claim 22 selected from

N-acetyl-Cys-(A)

Tyr-(A)

N-acetyl-Cys-Gly-(A)

Cys-(A)

where A is angiotensin I.

30. (Amended) An angiotensin derivative as claimed in [any one of claims 22 to 29] claim 22 which elicits a cross-reactive immune response with angiotensin I, angiotensin II, and/or angiotensinogen molecules.

31. (Amended) An angiotensin derivative as claimed in [any one of claims 22 to 30] claim 22 conjugated to a carrier.

34. (Amended) A method of combatting conditions associated with activation of the renin-angiotensin system comprising administering an angiotensin derivative as defined in [any one of claims 1-17] claim 5.

35. (Amended) A nucleic acid molecule coding for a linear angiotensin peptide derivative as claimed in [any one of claims 1-17] claim 5 and nucleic acid molecules with sequences complementary thereto.

38. (Amended) A method of combatting conditions associated with activation of the renin-angiotensin system comprising administering a nucleic acid molecule coding for a linear angiotensin peptide derivative as claimed in [any one of claims 1-16] claim 1 or an expression vector comprising a nucleic acid molecule coding for an angiotensin peptide derivative.

REMARKS

The foregoing amendments to the claims are made solely to remove multiple dependencies. No new matter has been added.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper.

However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

Karen Lee Orzechowski
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"Express Mail" Mailing Label Number Date of Deposit: December 22, 1999 I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box Patent Application, Assistant Commissioner for Patents, Washington, DC 20231. _____ Karen Lee Orzechowski	**CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner For Patents, Washington, DC 20231, on December 22, 1999. _____ Karen Lee Orzechowski

Attorney's Docket No. 41565/192844

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: James Francis GLOVER et al.
Appl. No.: Not yet assigned
Filed: December , 1999
Title: ANGIOTENSIN DERIVATIVES

STATEMENT CLAIMING SMALL ENTITY STATUS (37 C.F.R. § 1.9(f) & 1.27(c)) – SMALL BUSINESS CONCERN

I hereby declare that I am:

☐ the owner of the small business concern identified below:
☐ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF SMALL BUSINESS CONCERN: PROTEUS MOLECULAR DESIGN LIMITED

ADDRESS OF SMALL BUSINESS CONCERN: Beechfield House, Lyme Green Business Park, Macclesfield, Cheshire SK11 OJL Great Britain

I hereby state that the above-identified small business concern qualifies as a small business concern as defined in 13 C.F.R. 121., and reproduced in 37 C.F.R. § 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time, or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby state that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in the specification filed as a continuation of the United States designation of PCT International Application Number PCT/GB98/01833 filed on June 23, 1998, and having the title as listed above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern, or organization having rights in the invention must file separate statements as to their status as small entities, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under

Statement Claiming Small Entity Status
Page 2 of 2

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37 C.F.R. 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d), or a nonprofit organization under 37 C.F.R. § 1.9(e).

Each person, concern, or organization having any rights in the invention is listed below:

☐ no such person, concern, or organization exists.
☐ each such person, concern, or organization is listed below.

Name: _____

Address: _____

☐ Individual ☐ Small Business ☐ Nonprofit Organization

Name: _____

Address: _____

☐ Individual ☐ Small Business ☐ Nonprofit Organization

Separate statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b))

NAME OF PERSON SIGNING: Barrington Marshall Riley

TITLE OF PERSON OTHER THAN OWNER: Company Secretary

ADDRESS OF PERSON SIGNING: Proteus Molecular Design Macclesfield.

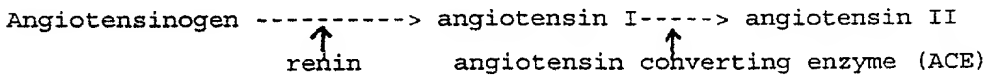
SIGNATURE: _____ DATE: 16 December 1999

WDC01/38905v3

ANGIOTENSIN DERIVATIVES

The present invention relates to analogues or derivatives of the mammalian peptide hormones angiotensin I and angiotensin II, and to immuno-therapeutic uses of these in particular for the therapy and prophylaxis of conditions associated with the renin activated angiotensin system.

Angiotensin peptides are involved in controlling arterial pressure in mammals. They are produced in several forms in the body as a result of a biochemical cascade known as the renin-angiotensin system (RAS), initiated by renin produced as a result of a fall in arterial pressure. In the RAS, represented schematically below, renin is released by the kidneys from stored pro-renin following a fall in arterial blood pressure, and acts enzymatically upon angiotensinogen to produce angiotensin I which is a decapeptide having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu. Two amino acids from the C-terminus of angiotensin I are rapidly cleaved, by angiotensin converting enzyme (ACE), present in the endothelium of the lungs, generally within 1-2 seconds, to produce the octapeptide angiotensin II, having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe.



Angiotensin I is very short lived within the body and has mild vasoconstrictor activity. Alone therefore it has insignificant effect on the circulatory system. Angiotensin II, however, is a vasoactive peptide which has a profound effect on the circulatory system, as well as on the endocrine system. Elevated levels of RAS-activated angiotensin II cause vasoconstriction and

renal retention of salt and water, both of which contribute to increased arterial pressure (hypertension) which can lead to cardiovascular damage. Angiotensin II has been implicated in a number of other disease states, including congestive heart failure. Hypertension is a major risk factor for heart attacks and strokes and congestive heart failure is the disease with the highest mortality within a few years of onset. There is a need for effective therapies for combatting these and other diseases associated with the renin-angiotensin system.

Current treatment for these diseases includes intervention in the RAS system using small organic molecules. One approach attempts to inhibit ACE with inhibitors such as lisinopril, captopril and enalapril, agents which are now established in management of hypertension. These drugs have not however been entirely successful. It seems that inhibition of ACE is only partial. Furthermore, because ACE lacks substrate specificity, biotransformation of other metabolically active peptides, including bradykinin may also be inhibited, which is undesirable. In addition, these drugs need to be taken on a regular basis, often for long periods, such as for the majority of adult life. A major drawback, however, of these drugs is their undesirable side effects, including dry cough and a first dose hypotensive effect with dizziness and possible fainting. Since anti-hypertensive therapies invariably need to be taken long term, e.g. for up to 30 years and sometimes even longer, these adverse side effects can result in loss of patient compliance, particularly in the absence of short term clinical benefit in a mainly asymptomatic condition, severely limiting the usefulness of this therapeutic approach.

A more recent therapeutic approach involves drugs which are angiotensin receptor antagonists which are intended to block the activity of angiotensin II. Examples include losartan and valsartan. The agents

which have been developed to date appear to be specific for only the AT_1 angiotensin receptor; they therefore block the dominant vasoconstrictor effects of angiotensin II, and are better tolerated but do not affect other actions of the angiotensin hormones. Experience with AT_1 receptor antagonists indicates that whilst they may be of comparable effectiveness to ACE inhibitors poor patient compliance remains a problem. There is accordingly a need for improved therapies of diseases associated with the RAS.

A potential approach in treating or preventing diseases or disorders associated with the activity of a hormone is to neutralise the effects of the hormone within the patient by immunotherapy i.e. by immunising the patient against the hormone such that the activity of the hormone is neutralised by specific anti-hormone antibodies. Such antibodies may be exogenously administered in passive immunisation or they may be generated *in situ* by active immunisation using an immunogen based on the hormone.

We have now developed new derivatives of angiotensin which are potent immunogens and which can be used in an immunotherapeutic approach to combat conditions associated with elevated levels of angiotensin II produced by the RAS.

In particular, derivatives of angiotensin have been developed in which one or more angiotensin peptides are coupled to a binder moiety, e.g. a peptide sequence, which facilitates attachment of the angiotensin peptide to an immunological carrier such as a protein or polypeptide to form an immunogenic conjugate capable in an immunised host of inducing antibodies which bind to angiotensin and neutralise its effects. These induced antibodies include those which may also bind to the precursor form, angiotensinogen and in this way, cleavage by renin to angiotensin I is prevented, thereby providing an additional blockade of the system. This

may be particularly relevant to reducing the effects of modulation of the negative feedback effects of Angiotensin II on renin production and release of Angiotensin I.

In one aspect, the present invention thus provides an angiotensin derivative comprising at least one angiotensin peptide moiety coupled to a peptide carrier-binding moiety.

These angiotensin derivatives may be used to immunise a patient against the hormone angiotensin II and/or its polypeptide precursor angiotensin I and/or angiotensinogen such that the activity of the hormone is neutralised by specific anti-hormone or antipolypeptide antibodies.

The angiotensin peptide moiety may be any moiety, without necessarily having the biological activity of a native angiotensin (ie. native hormone activity at the receptors, including both angiotensins I and II), in the body which is capable of acting as an immunomimic of native angiotensin peptides i.e. which immunologically mimics angiotensin so as to generate antibodies which bind to native angiotensin peptides. Thus, such a moiety may conveniently comprise an angiotensin peptide, preferably angiotensin I (a decapeptide of formula Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) or angiotensin II (an octapeptide of formula Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), or a functionally equivalent variant thereof. Such variants may include modifications of the angiotensin I or II sequence by single or multiple amino acid substitution, addition or deletion and also sequences where the amino acid residues are chemically modified, but which nonetheless retain angiotensin immunogenic activity. Such functionally (i.e. immunologically) equivalent variants may occur as natural biological variations, or they may be prepared using known and standard techniques for example by chemical synthesis or modification, mutagenesis, e.g.

site-directed or random mutagenesis etc. The important feature as regards the modification is that the angiotensin peptide retains the ability to act as immunomimic of native angiotensin. Thus for example, an amino acid may be replaced by another which preserves the physicochemical character of the angiotensin peptide or its epitope(s) e.g. in terms of charge density, hydrophilicity/hydrophobicity, size and configuration and hence preserve the immunological structure. "Addition" variants may include N- or C-terminal fusions as well as intrasequence insertion of single or multiple amino acids. Deletions may be intrasequence or may be truncation from the N- or C-termini. The term "angiotensin peptide" as used herein includes all native angiotensin peptides and their functionally equivalent variants.

The carrier-binding moiety serves as a means by which the angiotensin peptide moiety may be attached to an immunological carrier, which will generally be a protein or polypeptide, and thus preferably contains an amino acid residue having a reactive side chain, via which the angiotensin derivative may readily be coupled to the carrier using standard coupling techniques. Advantageously such a side chain may contain a free hydroxyl, carboxyl or thiol group. Such an amino acid may thus conveniently be a cysteine, tyrosine, aspartic acid or glutamic acid residue or a derivative thereof such as N-acetyl cysteine.

Angiotensin analogues of the invention have been shown to have improved coupling to an immunological carrier for inducing antibodies which can be used immunotherapeutically and these analogues have advantages in this regard over the native peptide.

The carrier-binding moiety may take the form of a peptide extension at the N- or C-terminal of an angiotensin peptide, or a peptide pendant from or disposed within a chain segment between two or more

angiotensin moieties.

Viewed from a further aspect, the present invention can be seen to provide an angiotensin derivative of Formula I

$$((A)\text{-}X_n)_m\text{-}L_p\text{-}Y\text{-}[L_q(X_r\text{-}(A))_s]_t \qquad (I)$$

wherein

A represents an angiotensin peptide moiety;

X represents an amino acid;

Y represents an amino acid having a side chain with a free -SH, -OH or -COOH group;

L represents an organic linker capable of binding a group $((A)\text{-}X_n)$- at one or more sites, e.g. capable of binding up to 10 $(A)X_n$ moieties;

n and r are each = 0-20;

m and s are each ≥1, e.g. 1 to 10, preferably 1, 2, 3 or 4; and

p, q and t are each 0 or 1;

with the proviso that if m≥2, then p=1, or if s≥2, then q=1.

Preferably A is an angiotensin peptide, and X may be attached at the N- or C-terminus of the angiotensin peptide.

Group L may be any organic linker structure, preferably however, it is a peptide chain, which may be linear or branched or a single amino acid residue, containing residues of natural or synthetic amino acids or pseudo-amino acids. However it may also represent a carboxyl- or amine-terminating dendritic or cascade polymer, for example a branched polyamine.

When t=0, it will be seen that the compounds of Formula (I) include derivatives wherein a carrier binding moiety (i.e. X-Y or X-L-Y) is attached at the N- or C-terminus of an angiotensin peptide, as a simple N- or C-terminal extension, or wherein multiple angiotensin peptide moieties are linked to a carrier-binding moiety terminating in a group Y, for example as a dendritic array or where the angiotensin moieties are attached at

multiple sites on the carrier-binding moiety.

When t=1, it will be seen that the derivatives may take the form of a "dimer"-type structure wherein the carrier-binding group Y of the carrier-binding moiety is disposed within a chain segment of the derivative i.e. effectively between two or more angiotensin peptide moieties.

If t=1, and L is an amino acid residue or a peptide chain, L may be or include a "chain-inverting" amino acid or pseudo amino acid (i.e. a compound capable of linking two peptide moieties, e.g. a diamine or dicarboxylic acid), this being a compound capable of inverting or reversing the N- to C-terminal direction of the peptide chain. Such a compound will thus generally include two amino or two carboxylic acid groups, e.g. glutamic acid or a α,ω-alkylene diamine or α,ω-alkylene dicarboxylic acid. When t=1, it is furthermore preferred that the total number of groups ((A)-X_n) - does not exceed 8.

Preferred compounds of Formula (I) include those wherein n and r are each 0-10, preferably 1-6, and those wherein m and s are each ≤8, preferably 1, 2 or 4.

Group X preferably represents an amino acid having no side chain or a hydrocarbyl side chain (preferably an alkyl, C_{3-7} cycloalkyl or cycloalkenyl, C_{3-7} cycloalkyl- or cycloalkenyl-alkyl, alkaryl, aralkyl or alkarylalkyl moiety in which each alkyl moiety may be saturated or unsaturated and contains up to 6 carbons and each aryl moiety is preferably a phenyl ring), particularly preferably an aliphatic side chain. Glycine, alanine, β-alanine, valine, leucine and isoleucine are preferred and glycine is especially preferred.

Group Y is preferably cysteine, tyrosine, glutamic acid or aspartic acid or a derivative thereof such as N-acetyl-cysteine.

Group L preferably contains at least one residue of an amino acid or pseudo amino acid containing at least

two amine or carboxyl groups e.g. lysine, arginine, glutamic acid or aspartic acid, particularly where t=0. Conveniently, such a preferred group L is a linear or branched peptide chain, eg. containing 2 to 15 amino acid residues. Branching may, of course, occur by peptide bond formation at an amine or carboxyl group of an amino acid residue side chain, eg. at a side chain amine group of lysine or arginine or at a side chain carboxyl group of aspartic or glutamic acid. A group L comprising one or more, eg. 1 to 3, lysine residues is especially preferred. Branching may occur by peptide bond formation at both the α-amino and e-amino groups of lysine.

Preferred compounds of Formula (I) thus include compounds of Formulae (II) to (IV):

$$(A)\text{-}X_n\text{-}Y \qquad (II)$$

$$(A)\text{-}X_n\text{-}L\text{-}Y \qquad (III)$$

$$((A)\text{-}X_n)_m\text{-}L\text{-}Y \qquad (IV)$$

$$(A)\text{-}X_n\text{-}L\text{-}Y\text{-}L\text{-}X_r\text{-}(A) \qquad (V)$$

wherein A, X, L, n and r are as hereinbefore defined and $m \geq 2$.

Where the compounds of Formula (IV) contain more than one (A) group, these are preferably attached at the same terminus i.e. preferably all are N-terminally or all are C-terminally attached.

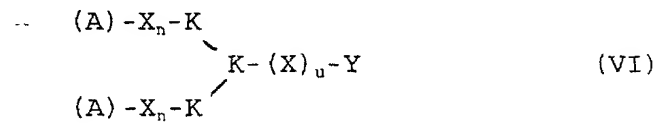
In compounds of Formulae (II) and (III) where X is C-terminally attached to a group A being an angiotensin peptide, Y is preferably cysteine. Where X is attached to the N-terminus of A, Y is preferably N-acetyl-cysteine.

In Formulae (II) to (V), X_n or X_r are each preferably chains of 1 to 6 glycine residues.

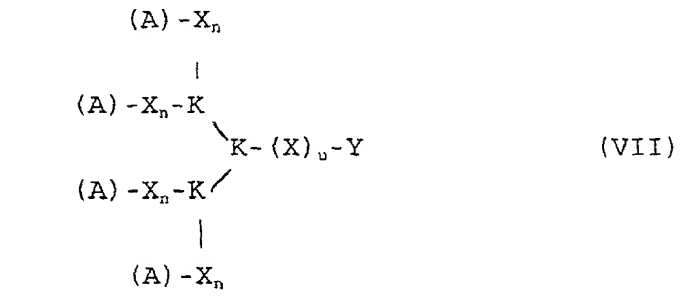
In compounds of formula (IV), m is preferably 2 or 4.

In Formulae (III) to (IV), L is preferably lysine, -lys-$(X)_u$, -lys-lys-$(X)_u$, or -lys-lys-$(X)_u$ with -lys branching from the second lys, wherein u is 0 to 10, preferably 0 to 6, and X is an amino acid as defined above.

Thus, preferred compounds of Formula (IV) are those of Formulae (VI) and (VII):



(VI)



(VII)

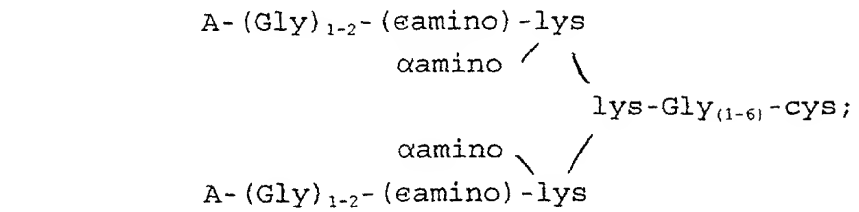
where A, X, Y, n and u are as hereinbefore defined, and K is lysine.

In the "dimer-type" derivatives of Formula (V) the angiotensin peptide moiety may preferably be a "reversed" or "inverted" sequence variant of an angiotensin peptide ie. an angiotensin peptide in which the order of the constituent amino acids is reversed.

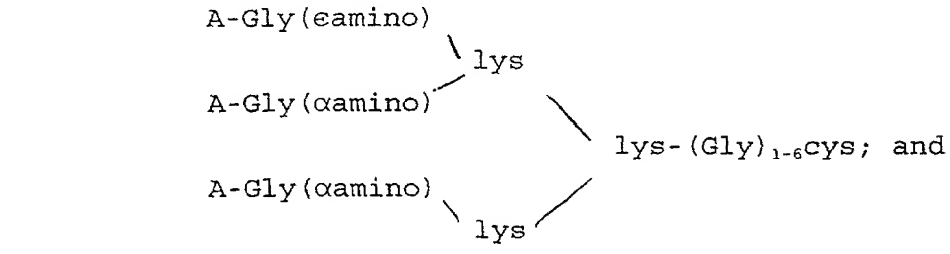
Representative angiotensin derivatives according to the invention include:

A-$(Gly)_{1-6}$-cys;

N-Acetylcys-$(Gly)_{1-6}$-A;



(The A-$(Gly)_{1-2}$-moiety may be bonded to either the α-amino or the ε-amino group)



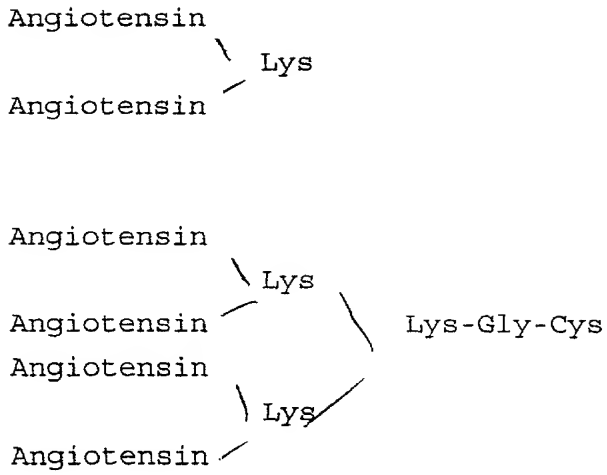
A'-$(Gly)_{1-6}$-cys-$(Gly)_{1-6}$-A;

N-acetyl-Cys-Ala-Angiotensin

N-acetyl-Cys-$(Ala)_4$-Angiotensin

N-acetyl-Cys$(Gly)_6$-Angiotensin

N-acetyl-Cys-Gly-Ala-Gly-Ala-Angiotensin



(A)-Gly Cys

(A)-Cys

(A)-Tyr

N-acetyl-Cys-(A)

Tyr-(A)

N-acetyl-Cys-Gly-(A)

Cys - (A)

wherein A is angiotensin I or angiotensin II and A' is angiotensin I or angiotensin II or an inverted or reverse angiotensin I or angiotensin II sequence.

Although Glycine is preferred, aliphatic side chain amino acids may be used in place of one or more of the Gly residues in the above formula.

Although the peptide analogues of the invention when examined by computer-aided energy minimisation

modelling are generally considered too small to be optimally immunogenic alone, it has been found that when coupled via the carrier-binding moiety to a carrier, these peptide analogues elicit a strong protective immune response. They are thus eminently suitable for use in immunotherapy against RAS-associated conditions. Without wishing to be bound by theory, it is believed that coupling of the peptides to a carrier by means of the carrier-binding moiety results in the analogues having substantially the same conformation as that of the native angiotensin peptides.

The new derivatives according to the invention may be generated using a number of standard techniques including, for peptides, the Merrifield solid phase method in which amino acids are added stepwise to a growing polypeptide linked to a solid matrix as described in R.B. Merrifield, Fed. Proc. Amer. Soc. Biol. (1962). 21, 412 and R.B. Merrifield, Jour. Amer. Chem. Soc. (1963), 85, 2149 and conventional FMOC chemistry. If desired, reactive side chain groups of the amino acids in the growing chain may be protected during the chain synthesis. Branched structures may be prepared by similar techniques.

Where the new derivatives are linear peptides these may also be prepared by recombinant DNA expression using techniques known in the art e.g. as described, for example, by Sambrook et al., in Molecular Cloning: A Laboratory Manual, Second Edition, 1989.

Thus the present invention also provides a nucleic acid molecule coding for the angiotensin peptide derivatives of the invention, and nucleic acid molecules with sequences complementary thereto.

According to a further aspect of the invention, we provide an expression vector comprising the said nucleic acid molecule of the invention. Such a vector may be suitable for expression in microorganisms which may be prokaryotic or eurkaryotic e.g. *E coli* or yeast, or in

plant or animal e.g. mammalian cells.

Such an expression vector, capable *in situ* of synthesising an angiotensin derivative according to the invention may also be used therapeutically and may be introduced to the subject in a variety of ways. Examples of these include topical application of the 'naked' nucleic acid vector in an appropriate vehicle for example in solution in a pharmaceutically acceptable excipient such as phosphate buffered saline (PBS), or administration of the vector by physical methods such as particle bombardment, also known as 'gene gun' technology, according to methods known in the art e.g. as described in US-5371015 in which inert particles, such as gold beads coated with the vector are accelerated at speeds sufficient to enable them to penetrate the skin surface, by means of discharge under high pressure from a projecting device.

Nucleic acid sequences encoding angiotensin derivatives of the invention may also be used immuno-therapeutically in the form of delivery vectors. These include viral delivery vectors, such as adenovirus or retrovirus delivery vectors known in the art into which the nucleic acid sequence is incorporated and which can be used for immunisation in ways known in the art.

Other non-viral delivery vectors which may be used to deliver the nucleic acid vectors of the invention include lipid delivery vectors, including liposome delivery vehicles, known in the art.

According to a yet further aspect, the present invention provides a host organism transformed with a vector according to the invention.

The angiotensin derivatives of the invention, as is the case for other small molecules, may be of insufficient size to stimulate antibody formation alone and may thus need to be conjugated to a macromolecular carrier in order to stimulate antibody production and a protective immune response.

Thus according to a further aspect, the present invention provides an angiotensin derivative as defined above conjugated to a carrier, preferably a polypeptide carrier.

Coupling of the derivative of the invention to the carrier may be by methods known in the art for example by treatment with heterobifunctional linking agents. Where coupling is via a terminal cysteine (or N-acetyl cysteine), the linking agent may be m-Maleimidobenzoyl-N-hydroxysulphosuccinamide ester; in which case maleimide modifies one or more lysine side chains in the peptide carrier, and a thioether bond forms at the terminal cysteine residue. Other coupling reagents known in the art, eg carbodiimide coupling, may also be used.

Any carrier known in the art for such purposes may be used, including the purified protein derivative of tuberculin, tetanus toxoid, diphtheria toxoid, keyhole limpet haemocyanin or derivatives thereof.

Where the angiotensin derivative is a linear peptide and the carrier is a protein or polypeptide, the entire peptide conjugate may also be made by recombinant DNA methods wherein a nucleic acid molecule encoding the conjugated molecule is expressed in an appropriate host cell.

The new angiotensin derivatives of the invention may be used in an immunotherapeutic approach to combatting diseases associated with normal or elevated levels of RAS activity and/or angiotensin peptides, and represents an advantageous method compared to currently available methods. Patient compliance should be increased in that less frequent dosing than is the case with current therapies is involved, and undesirable side effects are avoided.

Thus according to a further aspect, the present invention provides a pharmaceutical composition comprising an angiotensin derivative according to the

invention, or a conjugated angiotensin derivative according to the invention, together with one or more pharmaceutically acceptable carriers or excipients.

Viewed from a further aspect, the invention provides an angiotensin derivative according to the invention for use in therapy.

Viewed from a yet further aspect, the invention provides the use of an angiotensin derivative according to the invention in the manufacture of a medicament for use in combatting diseases associated with the renin-angiotensin system. Such diseases include congestive heart failure and hypertension such as systemic hypertension and other diseases in which the renin-angiotensin system contributes to the pathophysiology thereof, as well as diseases where the renin-angiotensin system has elevated levels of activity.

Viewed from a still yet further aspect, the invention provides a method of combatting conditions associated with the renin-angiotensin system comprising administering an angiotensin derivative according to the invention.

The method may be used to modulate blood pressure.

The angiotensin derivative according to the invention optionally conjugated to a carrier or recombinant nucleic acid encoding for the derivative may be administered by all conventional methods including parenterally (e.g. intraperitoneally, subcutaneously, intramuscularly, intradermally for example in the form of inert particles such as gold pellets or beads to which the derivative is adsorbed which may be accelerated at speeds sufficient to enable them to penetrate the skin of a subject, or intravenously), topically (e.g. as a cream to the skin), intra-articularly, mucosally (e.g. orally, nasally, vaginally, rectally and via the intra-ocular route) or by intrapulmonary delivery for example by means of devices designed to deliver the agents directly into the lungs

and bronchial system such as inhaling devices and nebulisers, and formulated according to conventional methods of pharmacy optionally with one or more pharmaceutically acceptable carriers or excipients, such as for example those described in Remingtons Pharmaceutical Sciences, ed. Gennaro, Mack Publishing Company, Pennsylvania, USA (1990).

Such compositions are conveniently formulated in unit dosage form e.g. for mucosal, parenteral or oral administration.

Actual treatment regimes or prophylactic regimes, formulations and dosages will depend to a large extent upon the individual patient and may be devised by the medical practitioner based on the individual circumstances.

The type of formulation will be appropriate to the route of administration. For example, parenteral administration by subcutaneous or intramuscular injection may be with a sterile aqueous suspension of the conjugated analogue in PBS, saline or water for injection, optionally together with one or more immunological adjuvants e.g. aluminium hydroxide, saponin, quil A, muramyl dipeptide, mineral or vegetable oils, vesicle-based adjuvants, non-ionic block co-polymers, or DEAE dextran. Additional components such as preservatives may be used.

The dosage for injection may be in the range 1-100 μg peptide equivalent and the frequency of administration may be upwards of from once every three or six months, to once every year or once every five years.

For oral administration, the conjugated derivatives may be formulated as tablets, liquid, capsules etc. Dosages range from 1 to 1000 μg peptide equivalent with dosing occurring at intervals dependent on bioavailability of product.

According to a still yet further aspect, the

present invention provides a method for achieving maximal blockade of angiotensin hormones comparable to or exceeding that achieved by existing therapies based on ACE inhibitors and/or angiotensin II receptor antagonists, said method comprising administering an angiotensin derivative according to the invention.

The invention will now be described in further detail in the following non-limiting Examples, with reference to the drawings in which:

Figure 1 is a graph showing antibody titres +/- sem, n=6 (dilution corresponding to 0.1 increase in OD) against time (sample day);

A = Control

B = Derivative 3 of Example 2

C = Derivative 1 of Example 2

D = Derivative 4 of Example 2

E = Derivative 2 of Example 2.

Figure 2 is a graph showing peak change in blood pressure following administration of A1 in control rats and in rats immunised with a conjugate of an analogue of A1 in groups C and J of Example 4.

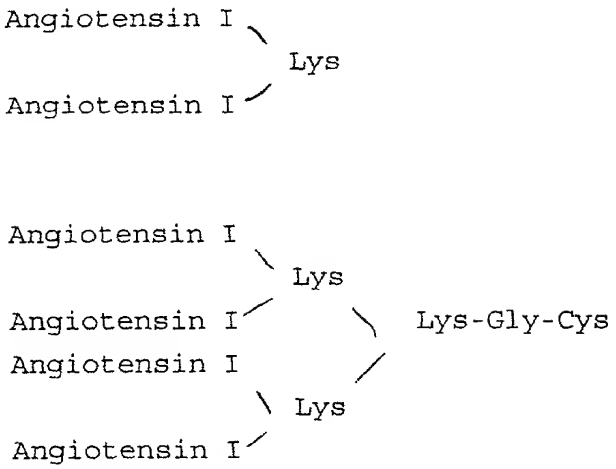
Figure 3 shows recordings of mean blood pressure changes in response to A1 in animals of groups A and C of Example 4.

Figures 4, 5 and 6 are bar charts showing antibody titres measured in terms of A^{450} in an ELISA assay using in the assay in Figure 4 angiotension I, in Figure 5 angiotensin II and in Figure 6 angiotensinogen, the ELISAs showing the binding of partially purified rat antisera raised against vaccines containing analogues of angiotensin hormones.

Figures 7 and 8 are graphs showing antibody titres against time (sample day) for the following derivatives

N-acetyl-Cys-$(Ala)_4$-Angiotensin I

N-acetyl-Cys(Gly)$_6$-Angiotensin I



Example 1: Peptide generation.

Peptides were synthesised by the Fmoc strategy of solid phase peptide synthesis on a Protein Technologies, Symphony Peptide Synthesiser. The resin used was Tentagel S-NH2 with a Rink Amide linker. The side chain protecting groups of the Fmoc amino acids used were Trt for Cys His, Asn and Gln, tBu for Tyr Thr, Asp, Glu and Ser; Boc for Lys and the indole N of Trp, Pmc for Arg. Activation of the carboxyl groups was achieved using, TBTU/HOBt/DIPEA, all couplings were carried out in DMF. Deprotection of the Fmoc groups was achieved with 20% Piperidine in DMF. Cleavage of the peptides from the resin was carried out with 5%Anisole/5%Thioanisole/ 5%EDT/3%Water/2%TES in TFA for 1 hour. The peptides were purified by RP-HPLC using a 40mm x 210mm Deltapak C18 radial compression column on a Waters Delta prep 4000 and characterised by MALDI-TOF on a Kratos Maldi 3 and by AAA.

For dendrimers Fmoc Lys(Fmoc)-OH is attached by the

methods above and gives both α and ε amino groups free for peptide elongation. Quantities of Fmoc amino acids used have to be increased accordingly.

Rink Amide Linker = p-[(R,S--[1-(9H-Fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid
Fmoc =9-Fluorenylmethoxycarbonyl
Trt = Trityl, Triphenylmethyl
tBu = tertiary butyl
Boc = tertiary butyloxycarbonyl
Pmc = 2,2,5,7,8-Pentamethylchroman-6-sulphonyl
TBTU = 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
HOBt = N-Hydroxybenzotriazole
DIPEA = Diisopropylethylamine
DMF = N,N Dimethylformamide
EDT = Ethanedithiol
TES = Triethylsilane
TFA = Trifluoroacetic acid
RP-HPLC = Reverse phase high performance liquid chromatography
MALDI-TOF = Matrix assisted laser desorption ionisation - time of flight
AAA = Amino acid analysis
Fmoc-Lys(Fmoc)-OH = α, ε di-9-fluorenylmethoxycarbonyl lysine

The following peptides were synthesized in this manner:

(1) Angiotensin I-gly-cys	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu Gly-Cys
(2) Angiotensin II-gly-cys	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-Gly-Cys
(3) N-acetyl-Cys-Gly-Angiotensin I	N-acetyl-Cys-Gly-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
(4) N-acetyl-Cys-Gly-Angiotensin II	N-acetyl-Cys-Gly-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

Example 2: Conjugation procedure.

To tetanus toxoid solution in phosphate buffered saline (PBS), a 60 molar excess of S-MBS, m-Maleimidobenzoyl-N-hydroxysulphosuccinimide ester is added and stirred for 2 hours at 4°C in a sealed vial.

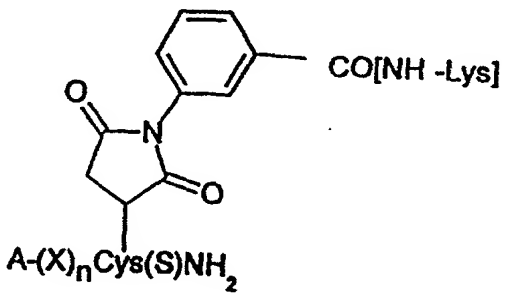
Excess S-MBS crosslinker is removed by chromatography (gel exclusion, PD-10, G-25 sephadex column) in PBS. The activated tetanus toxoid peak is collected, assayed for free maleimido groups and used as below.

The resulting carrier protein solution is purged with N_2, and a 12 molar excess of angiotensin derivative peptide added. The resulting solution is stirred for 4 hours at 20°C in a sealed container.

The conjugate is purified from free peptide by gel exclusion chromatography as above. A final assay for loss of free maleimido groups is performed on a sample of the mixture to prove that all available sites are conjugated.

The final conjugate is diluted to a working concentration and formulated as desired.

The structure of S-MBS crosslinked conjugate with a linear C-terminal extended angiotensin peptide derivative (eg. derivatives (1) and (2) of Example 1) is shown below:



Following this procedure, angiotensin derivatives (1) to (4) of Example 1 were conjugated individually to aliquots of tetanus toxoid.

Example 3: Immunisation Studies.

The four angiotensin derivatives of Example 1, conjugated individually to aliquots of tetanus toxoid as described in Example 2, were formulated by adsorption to 0.4% (w/v) aluminium hydroxide gel (Alhydrogel, Superfos s/a, Denmark) in a normal saline (0.9% (w/v)) vehicle.

All conjugates were used as 10μg/ml peptide equivalent solution.

Male Sprague-Dawley rats were used in 5 treatment groups, with 6 rats per group.

The treatment groups received:

Vehicle, sterile saline	0.5 ml/rat
N-acetyl-cys-gly-Angiotensin I derivative immunotherapeutic	5 μg peptide equivalent/ rat in 0.5ml vehicle
Angiotensin I-gly-cys derivative immunotherapeutic	5 μg peptide equivalent/ rat in 0.5 ml vehicle
N-acetyl-cys-gly-Angiotensin II derivative immunotherapeutic	5 μg peptide equivalent/ rat in 0.5 ml vehicle
Angiotensin II-gly-cys derivative immunotherapeutic	5 μg peptide equivalent/ rat

The route used was subcutaneous and each rat received 3 separate doses of the specified test article during the course of the study.

The bodyweight of each rat is recorded once a week throughout the experimental procedure.

Experimental procedure

In this initial investigation the core temperature of each rat was recorded, as part of the general physiological monitoring of the animals.

On day 1 and subsequently on days 22 and 43, the rats received a single subcutaneous dose of the vehicle, or the test articles. Twenty four hours following each administration (days 2, 23 and 44) and on further days specified in table 1 a venous blood sample (0.5 ml) was subsequently taken while the rat was restrained.

Each sample of venous blood was collected in a glass tube, cooled on ice and allowed to clot, then centrifuged to yield serum within 45 minutes of sampling. Serum samples were frozen at approximately -20°C as soon as possible.

Table 1: Time Schedule of Study Procedures

Week	Day	Treatment	Blood Sample
1	1	Test articles, vehicle	
	2	No dosing	+
2	9	No dosing	+
	16	No dosing	+
3	22	Test articles, vehicle	
	23	No dosing	+
4	30	No dosing	+
5	37	No dosing	+
6	43	Test articles, vehicle	
	44	No dosing	+
7	51	No dosing	+
8	58	No dosing	+
9	65	No dosing	+
10	72	No dosing	+
11	79	No dosing	+
12	86	No dosing	+

Each serum sample was assayed for the generation by the treatment of an antibody response by titration of anti-angiotensin peptide-antibodies present in the sera by Enzyme Linked ImmunoSorbant Assay (ELISA).

This assay was performed as follows:

Coat the 96 well uniwell microtitre plates with 50 µl detection substrate e.g. Angiotensin II-Gly-Cys-BSA (10 µg peptide equivalent/well) for 1 hour at room temperature. At the same time place 50 µl PBS into separate wells to act as a substrate blank.

Wash the plates 3 times with 200 µl Phosphate Buffered Saline (PBS)/0.1% Tween 20.

Add 200 µl/well of 3% (w/v) milk powder (Marvel) in PBS and leave for 1 hour at room temperature to block non-specific antibody binding.

Wash the plates 3 times with 200 µl PBS/ 0.1% Tween 20.

Dilute the serum samples to a suitable dilution with PBS. Typical dilutions would be as follows:

i) 1/100 - 5 µl rats sera + 495 µl PBS
ii) 1/1000 - 20 µl (i) + 180 µl PBS
iii) 1/2000 - 10 µl (i) + 190 µl PBS
iv) 1/5000 - 4 µl (i) + 196 µl PBS

Load the appropriate diluted sera (50 µl) to appropriate wells and incubate at 20°C for 1 hour to permit substrate:antibody binding.

Wash the plates 3 times with 200 µl PBS/0.1% Tween 20.

Dilute rabbit anti-rat IgG peroxidase conjugate 1:5000 in PBS i.e. 1 μl IgG peroxidase + 5 mls PBS. This binds to the rat serum antibody and allows antibody detection.

Add 50 μl of the diluted IgG peroxidase to the appropriate wells and leave for 45 minutes at room temperature.

Wash the plates 3 times with 200 μl PBS.

250 μl aliquot of the perodidase substrate $3,3^1,5,5^1$,-tetra methyl benzidine (TMB) to 25 mls 0.1M sodium acetate buffer pH5.5 with 4 μl 30% hydrogen peroxide.

Add 100 μl of the prepared TMB substrate to the appropriate wells, including the blank wells. A colour producing reaction occurs where antibody/ substrate binding has occurred. Leave for 15 minutes at room temperature, then terminate the reaction with 50 μl 10% sulphuric acid added to each well.

The plate was read for absorbance of light at 405 nm generated by the reaction of the peroxidase enzyme on the TMB substrate and is proportional to the amount of primary (anti-angiotensin) antibody bond. Results for the 4 sample conjugate formulations of derivatives (1) to (4) of Example 1 are shown in Figure 1.

Figure 1 shows a time course of mean antibody titre (+/- Sem, n=6) on the y axis at different sample times, measured in days on the x axis. The titre is the SAS estimated dilution of ser [illegible] required for a 0.1 OD change from baseline levels in the ELISA assay.

The changes in antibody levels against angiotensin peptides can be seen over time, and are summarised below:

Immunogen	Peak titre	Day	Terminal titre (Day 86)
(B) N-acetyl-cys-gly-Angiotensin I	12,218 ± 3576	86	As peak
(C) Angiotensin I-gly-cys	9,535 ± 4423	30	5068 ± 2038
(D) N-acetyl-cys-gly-Angiotensin II	15,726 ± 8271	30	10,239 ± 6544
(E) Angiotensin II-gly-cys	5090 ± 2965	37	2011 ± 1250

(A) is the control

In parallel with the antibody titre data, all animals were examined for gross physiological changes in body temperature, weight and general appearance, as an overall assessment of toxic or harmful effects.

No adverse effects were recorded on any of the 4 angiotensin immunoconjugate treatment groups, showing that the treatments are effective in generating anti-angiotensin antibodies, without harmful physiological effects in the animals.

Example 4: Effects of active immunisation against angiotensin peptides on the pressor effects of exogenous angiotensin I (AI) in conscious rats

In this experiment to demonstrate the potential of active immunisation with angiotensin analogues, certain analogues of angiotensin I (AI) and angiotensin II (AII) were conjugated to carrier proteins which are good immunogens. These immunoconjugates were adjuvanted and shown in immunised rats to generate a strong anti-angiotensin immune response.

The immunised rats were examined with regard to inhibition of the pressor response to exogenous AI.

Materials and Methods

Angiotensin immunotherapeutic vaccine preparation

The angiotensin analogues used in this study were:

AI analogue is: N-acetyl-cysteine-glycine-angiotensin I
AII analogue is: N-acetyl-cysteine-glycine-angiotensin II

The analogues of AI and AII were prepared using a Symphony peptide synthesiser (Anachem).

The conjugation carrier proteins, tetanus toxoid (TT) (Chiron Behring, GmbH), keyhole limpet haemocyanin (KLH) (Biosyn, GmbH) and non toxic recombinant diphtheria toxin (DT) (Chiron Behring, GmbH), were activated using a suitable bivalent linker. The 'activated' carrier protein was separated from the excess cross-linker reagent by size exclusion chromatography.

The following conjugates were made

Sample Group	Conjugate
A	Saline control
B	AII analogue, TT carrier protein
C	AI analogue, TT carrier protein
D	AII analogue, DT carrier protein
E	AII analogue, KLH carrier protein
F	AII analogue, TT carrier protein
G	equal mix of AI and AII analogues TT carrier protein
H	AII analogue, TT carrier protein
J	AI analogue, TT carrier protein
K	AII analogue, TT carrier protein
L	AI analogue, TT carrier protein

Key: AI/AII Peptide analogues of angiotensin hormones
TT Tetanus toxoid
DT non-toxic recombinant Diphtheria toxin
KLH Keyhole Limpet Haemocyanin

An excess of the AI and/or AII analogues was mixed with the activated carrier proteins and allowed to react, after which AI/AII-carrier protein conjugates were separated from the remaining free analogue by size exclusion chromatography.

The conjugates were sterilised by filtration through a 0.2 μm filter (Millipore) and formulated with adjuvant and saline vehicle to yield the appropriate vaccine for administration.

Alhydrogel® (Superfos S.A.) was the chosen aluminium hydroxide gel adjuvant for this study and 0.9% saline (Flowfusor®, Fresenius) the vaccine vehicle.

Table 2 shows the conjugate formulations administered to each of the treatment groups. The conjugates were formulated with aluminium hydroxide adjuvant, other than the conjugate of Group F which was formulated with DEAE (diethylaminoethyl)-dextran adjuvant.

Immunisation and AI Challenge

Male, Sprague Dawley rats (initially 200-250 g: Harlan Olac: n=6 for all groups) were injected (0.5 ml, sc.) with saline or immunotherapeutic vaccines on the days specified in Table 2.

On day 61, under sodium methohexitone anaesthesia (40-60 mg kg^{-1} i.p., supplemented as required), catheters were implanted in the distal abdominal aorta (via the ventral

caudal artery) and right jugular vein. The following day, conscious rats were given increasing i.v. bolus (0.1 ml) doses of AI (3-60 pmol rat^{-1}), while mean systemic arterial blood pressure and heart rate were recorded. At the end of the experiment animals were given i.v. sodium pentobarbitone (100 mg) and a blood sample was taken by cardiac puncture for the measurement of anti-angiotensin antibodies by ELISA.

Table 2
Treatment regime, formulations, doses, injection frequency and experimental regimes for study

		Injections					Catheters	Challenge (AI)
	Days	0	14	21	28	42	61	62
Group	**Formulation**	**Vol/Dose**						
A	Saline Control	0.2 ml	X		X		X	X	X
B	AII analogue, TT carrier protein, AlOH adjuvant	5 μg	X		X		X	X	X
C	AI analogue, TT carrier protein, AlOH adjuvant	5 μg	X		X		X	X	X
D	AII analogue, DT carrier protein, AlOH adjuvant	5 μg	X		X		X	X	X
E	AII analogue, KLH carrier protein, AlOH adjuvant	5 μg	X		X		X	X	X
F	AII analogue, TT carrier protein, DEAE adjuvant	5 μg	X		X		X	X	X
G	equal mix of AI and AII analogues TT carrier protein, AlOH adjuvant	2x2.5 μg	X		X		X	X	X
H	AII analogue, TT carrier protein, AlOH adjuvant	25 μg	X		X		X	X	X
J	AI analogue, TT carrier protein, AlOH adjuvant	25 μg	X		X		X	X	X
K	AII analogue, TT carrier protein, AlOH adjuvant	5 μg	X	X		X		X	X
L	AI analogue, TT carrier protein, AlOH adjuvant	5 μg	X	X		X		X	X

Key:
AI/AII Peptide analogues of angiotensin hormones
TT Tetanus toxoid
DT non-toxic recombinant Diphtheria toxin
KLH Keyhole Limpet Haemocyanin
DEAE Diethylaminoethyl cellulose
AlOH Aluminium hydroxide gel

Angiotensin analogue antibody ELISA

ELISA plate wells (Anachem) were coated with 10μg peptide equivalents of either AI or AII conjugated to bovine serum albumin (BSA) as a carrier.

The coated wells were washed with PBS (0.2% w/v)/Tween (Sigma) and blocked with 3% Marvel before diluted sera from the vaccinated rats were incubated in their respective wells. The sera had been diluted in PBS (Sigma) over a range from 2,500-20,000 fold.

Immobilised antibodies were detected in the wells using a rabbit anti-rat IgG/horseradish peroxidase conjugate and revealed using 3,3'-5,5'-tetra-methyl benzidine with H_2O_2 (Sigma). The reaction was terminated after 15 min at 22°C by the addition of 10% (v/v) H_2SO_4 (Sigma).

Colour generated was determined by absorbance at 450nm using a Packard plate reader. The resultant absorbance readings were analysed by a statistical package (SAS Institute 1997) to determine titre.

Statistical analysis of blood pressure changes on AI Challenge

The maximum change in mean blood pressure and heart rate over their immediate pre-challenge values were calculated for each animal and each challenge dose. Differences between treated groups and unimmunised controls were assessed by ANOVA using Dunnett's test.

Dose response analysis

The main effect of immunisation was to cause a parallel shift in the blood pressure dose response of animals to AI challenge. To estimate the size of this shift, a

logistic model was derived and fitted to the dose response:

$$\Delta BP = \frac{\Delta BP_{max}}{1+(d\ /ED50)^{-\alpha}} + \epsilon \qquad \epsilon \sim N(0,\sigma^2)$$

where d is the dose of AI, BP_{max} is the maximal change in blood pressure, α a shape parameter and ED_{50} is the dose of AI giving a half maximal response. Separate ED_{50} estimates were obtained for each animal. Significant differences between treatment groups and unimmunised controls were assessed by ANOVA of log-transformed ED_{50} values using Dunnett's multiple comparison test.

Results

Table 3 summarises some of the results, showing that active immunisation caused significant shifts in the pressor dose-response to AI and marked increases in antibody titres.

Clear effects on blood pressure are demonstrated with these treatments and the maximum dose shift (8.9x the control) are seen with a conjugate containing the AI analogue and tetanus toxoid on an aluminium hydroxide adjuvant.

Table 3 also demonstrates the relationship between anti-angiotensin antibody titre and response. In general, it can be seen that there is broad agreement between treatment induced titre and mean treatment induced dose shift, but no obvious dose response between groups C and J is apparent.

Figures 2 and 3 illustrate the results for control rats (Group A) and rats immunised with a conjugate of the AI

analogue and tetanus toxoid, presented on an AlOH gel adjuvant at a peptide equivalent dose of 5μg (low; Group C) and 25μg (high; Group J).

Figure 2 is a graph showing pressor effects of AI in control rats (Group A) and rats immunised with AI analogue at a dose of 5 μg (low, Group C) or 25 μg (High, Group J). The y axis shows peak change in BP (mm Hg) and the x axis shows the angiotensin I dose (pmol/ rat) in control, high dose group J (25 μg) and low dose group C (5 μg) animals. Errors bars shows 95% confidence interval on mean, based on pooled within group standard deviation (n=6), shown for control group only.

Figure 3 shows recordings of mean blood pressure changes in response to AI (3, 18 and 60 pmol bolus dose) in representative animals from group A(control) and Group C (5 μg dose).

Conclusion

Treatment with a conjugate containing an Al analogue and tetanus toxoid on an aluminium hydroxide adjuvant gives a highly significant reduction in the pressor response to exogenous Al.

Table 3

Treatment	Median ED_{50}	Mean treatment-induced dose shift	Anti-angiotensin antibody titre, ± s.e. mean (n=6)
A	8.9	-	0 0
B	39.6	4.5*	15300 ± 2100
C	79.1	8.9***	32100 ± 7800
D	19.6	2.2	9200 ± 2200

E	17.6	2.0	4700 ± 600
F	15.2	1.7	5500 ± 700
G	24.5	2.8	8300 ± 2000
H	38.2	4.3*	12100 ± 2500
J	74.7	8.4***	20100 ± 2300
K	13.9	1.6	5000 ± 900
L	43.0	4.8*	26100 ± 9400

Median AI bolus ($pmol.rat^{-1}$) to achieve half-maximal increase in mean blood pressure (ED_{50}) and corresponding anti-angiotensin antibody titres in control (group A) and immunised (groups B-L) rats. Significance probabilities adjusted for multiple comparisons by Dunnett's method (*=P<0.05, **=P<0.01, ***=P<0.001).

Example 5: Characterisation of antibodies produced in Example 3

Antibodies produced in Example 3 were enriched by affinity chromatography as follows:

Materials

1 mL HiTrap protein G affinity column (Pharmacia Biotech: 17-0404-03)
Wash buffer (WB) = PBS pH 7.2
Elution buffer (EB) = 0.1M glycine (HCL) pH 2.7
Neutralizing buffer (NB) = 1M Tris (HCL) pH 9
Storage buffer (SB) = 20% ethanol (v/v)

1. Rat sera from terminal bleeds following inoculation with each of TT-NAc-CG-angiotensin I (5 mL), angiotensin I-GC-TT (7 mL), TT-NAc-CG-angiotensin II (6 mL) or angiotensin II-GC-TT (5 mL), were clarified by centrifugation, filtered through a 1 μm PTFE disc filter then dialyzed against PBS pH

7.2. Each was then separately enriched as follows.

2. The HiTrap column was washed and equilibrated with 5 mL of WB.

3. Prepared sera (Point 1) was passed once through the HiTrap column, the waste was collected and stored at -20°C.

4. The HiTrap column was washed with 5 mL of WB to remove any remaining waste sera.

5. Immobilized antibodies were eluted using 10 mL of EB. The eluent was collected in 1 ml fractions each being immediately neutralized with 0.1 mL of NB.

6. At the end of the run the HiTrap column was washed with SB and stored at 4°C.

ELISA assays were carried out as described in Example 3 using plates coated as follows:

1) for angiotensin I and II

Materials:

Nunc Maxisorp ELISA plates (Life Technologies: 430341A).
Human Angiotensin I (Bachem: H-1680)
Human Angiotensin II (Bachem: H-1705)
0.1M carbonate buffer, pH 9.8 (0.316g Na_2CO_3 & 0.584g $NaHCO_3$ per 0.1 l)

Other materials used were as in the ELISA method detailed in Example 3 above.

1. 100 µL of either angiotensin I or angiotensin II,

depending on the specific binding event to be measured @ 0.2 mg/ml of 0.1M carbonate buffer, pH 9.8) was added to a suitable number of ELISA plate wells, and incubated for 1 hour at 22°C.

2. The ELISA plate was washed with PBS/Tween, blocked with Marvel then washed with PBS/Tween again as by the ELISA method described in Example 3 above.

3. The enriched antibodies from sera raised to Tetanus toxoid (TT) conjugates TT-NAc-CG-Ang I, Ang I-GC-TT, TT-NAc-CG-Ang II and Ang II-GC-TT, were incubated (1 hour, 22°C) in the coated wells at 2.5 μg/ml of PBS pH 7.2.

The ELISA was then completed as by the method described in Example 3 but absorbance was read at 450 nm.

2) For angiotensinogen:

The ELISA for detection of native angiotensinogen (Sigma: A-2562) was performed by the method of Example 3.

The enriched antibodies from sera raised to Tetanus Toxoid (TT) conjugates TT-NAc-CG-Ang I, Ang I-GC-TT, TT-NAc-CG-Ang II and Ang II-GC-TT were incubated (1 hour 22°C) at 0.5 μg/ml of PBS pH 7.2.

Results are shown in Figures 4, 5 and 6 which show absorbance read with ELISA plates coated with angiotensin I (Fig. 4), angiotensin II (Fig. 5) and angiotensinogen (Fig. 6) and shows that antibodies raised to each of the angiotensin derivatives conjugated to TT ie TT-NAc-CG-Ang I, Ang I-GC-TT, TT-NAc-CG-Ang II and Ang II-GC-TT recognised angiotensin I, angiotensin II and angiotensinogen.

Example 6: Generation of further angiotensin derivatives and immunisation studies

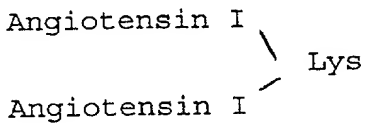
The following angiotensin derivative peptides 1 - 6 were synthesised according to the method of Example 1

N-acetyl-Cys-Ala-Angiotensin I (1)

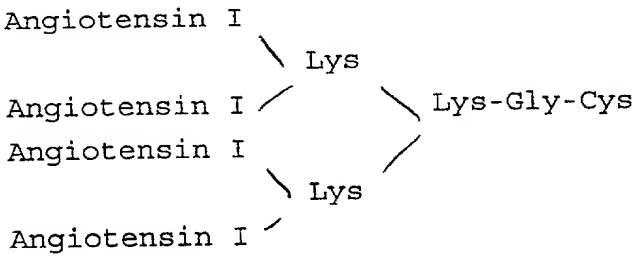
N-acetyl-Cys-$(Ala)_4$-Angiotensin I (2)

N-acetyl-Cys$(Gly)_6$-Angiotensin I (3)

N-acetyl-Cys-Gly-Ala-Gly-Ala-Angiotensin I (4)



(5)



(6)

These peptides were conjugated to tetanus toxoid as described in Example 2, and formulated for immunisation studies using the protocol described in Example 3.

Antibody titres were measured using the ELISA technique described in Example 4 against the peptides used in the immunogen.

Results are shown in Figures 7 and 8 which are graphs showing, on the y axis the antibody titre, as the

dilution factor to produce a SAS™ (Statistics Analysis System) designated 0.1 OD unit change, at various sample days. Each data point shown represents the mean of 5 serum samples from 5 different animals, each assayed in duplicate.

All peptides were shown to be effective in generating an anti-angiotensin I response. The responses varied in extent and duration.

Claims

1. The use of an angiotensin derivative comprising at least one angiotensin peptide moiety coupled to a peptide carrier-binding moiety in the manufacture of a medicament for use in combatting diseases associated with the renin-angiotensin system.

2. The use as claimed in claim 1 wherein the angiotensin moiety comprises angiotensin I or angiotensin II or a functional equivalent of angiotensin I or angiotensin II.

3. The use as claimed in claim 1 or claim 2 wherein the carrier binding moiety contains an amino acid residue having a reactive side chain.

4. The use as claimed in any one of the preceding claims wherein the carrier binding moiety is a peptide extension at the N- or the C-terminus of an angiotensin peptide moiety.

5. The use as claimed in any one of the preceding claims wherein the angiotensin derivative is of Formula I

$$((A)\text{-}X_n)_m\text{-}L_p\text{-}Y\text{-}[L_q(X_r\text{-}(A))_s]_t \qquad (I)$$

wherein

A represents an angiotensin peptide moiety;

X represents an amino acid;

Y represents an amino acid having a side chain with a free -SH, -OH or -COOH group;

L represents an organic linker capable of binding a group $((A)\text{-}X_n)$- at one or more sites, e.g. capable of binding up to 10 $(A)X_n$ moieties;

n and r are each = 0-20;

m and s are each ≥1, e.g. 1 to 10, preferably 1, 2, 3 or 4; and

p, q and t are each 0 or 1;

wherein X may be attached at the N- or C-terminus of the angiotensin peptide moiety with the proviso that if $m \geq 2$, then p=1, or if $s \geq 2$, then q=1.

6. The use as claimed in claim 5 wherein A is an angiotensin peptide.

7. The use as claimed in any one of claims 5 or 6 wherein L is a peptide chain.

8. The use as claimed in any one of claims 5 to 7 wherein n and r are each 0-10.

9. The use as claimed in any one of claims 5 to 8 wherein m and s are each ≤ 8.

10. The use as claimed in any one of claims 5 to 9 wherein X is an amino acid having no side chain or a hydrocarbyl side chain (preferably an alkyl, C_{3-7} cycloalkyl or cycloalkenyl, C_{3-7} cycloalkyl- or cycloalkenyl-alkyl, alkaryl, aralkyl or alkarylalkyl moiety in which each alkyl moiety may be saturated or unsaturated and contains up to 6 carbons and each aryl moiety is preferably a phenyl ring), particularly preferably an aliphatic side chain.

11. The use as claimed in any one of claims 5 to 10 wherein X is glycine, alanine, β-alanine, valine, leucine or isoleucine.

12. The use as claimed in any one of claims 5 to 11 wherein the angiotensin derivative is selected from

$$(A)\text{-}X_n\text{-}Y \qquad (II)$$

$$(A)\text{-}X_n\text{-}L\text{-}Y \qquad (III)$$

$((A)-X_n)_m-L-Y$ (IV)

$(A)-X_n-L-Y-L-X_r-(A)$ (V)

wherein A, X, L, n and r are as hereinbefore defined and $m \geq 2$.

13. The use as claimed in any one of the preceding claims wherein the angiotensin derivative is selected from

(A)-Gly Cys

(A)-Cys

(A)-Tyr

N-acetyl-Cys-(A)

Tyr-(A)

N-acetyl-Cys-Gly-(A)

Cys - (A)

(A) - N-acetyl-Cys

where A is angiotensin I or II.

14. The use as claimed in any one of the preceding claims wherein the angiotensin derivative elicits a cross-reactive immune response with angiotensin I, angiotensin II, and/or angiotensinogen molecules.

15. The use as claimed in any one of the preceding claims wherein the angiotensin derivative is conjugated to a carrier.

16. The use as claimed in claim 15 wherein said carrier is a polypeptide.

17. The use as claimed in claim 16 wherein the carrier is selected from the purified protein derivative of tuberculin, tetanus toxoid, diphtheria toxoid, keyhole limpet haemocyanin or derivatives thereof.

18. The use as claimed in any one of the preceding claims wherein said disease is congestive heart failure or hypertension.

19. The use as claimed in claim 18 for the modulation of blood pressure.

20. A pharmaceutical composition comprising an angiotensin derivative as defined in any one of claims 1-14, or a conjugated angiotensin derivative as defined in any of claims 15 to 17 , together with one or more pharmaceutically acceptable carriers or excipients.

21. An angiotensin derivative as defined in any one of claims 1-17 for use in therapy.

22. An angiotensin derivative of Formula I

$$((A)\text{-}X_n)_m\text{-}L_p\text{-}Y\text{-}[L_q(X_r\text{-}(A))_s]_t \qquad (I)$$

wherein

A represents an angiotensin I peptide moiety;

X represents an amino acid;

Y represents an amino acid having a side chain with a free -SH, -OH or -COOH group;

L represents an organic linker capable of binding a group $((A)\text{-}X_n)$- at one or more sites, e.g. capable of binding up to 10 $(A)X_n$ moieties;

n and r are each = 0-20;

m and s are each ≥1, e.g. 1 to 10, preferably 1, 2, 3 or 4; and

p, q and t are each 0 or 1;

wherein X is attached at the N-terminus of the angiotensin peptide moiety with the proviso that if $m \geq 2$, then p=1, or if $s \geq 2$, then q=1.

23. An angiotensin derivative as claimed in any one of claim 22 wherein L is a peptide chain.

24. An angiotensin derivative as claimed in any one of claims 22 or 23 wherein n and r are each 0-10.

25. An angiotensin derivative as claimed in any one of claims 22 to 24 wherein m and s are each ≤ 8.

26. An angiotensin derivative as claimed in any one of claims 22 to 25 wherein X is an amino acid having no side chain or a hydrocarbyl side chain (preferably an alkyl, C_{3-7} cycloalkyl or cycloalkenyl, C_{3-7} cycloalkyl- or cycloalkenyl-alkyl, alkaryl, aralkyl or alkarylalkyl moiety in which each alkyl moiety may be saturated or unsaturated and contains up to 6 carbons and each aryl moiety is preferably a phenyl ring), particularly preferably an aliphatic side chain.

27. An angiotensin as claimed in any one of claims 22 to 26 wherein X is glycine, alanine, β-alanine, valine, leucine or isoleucine.

28. An angiotensin derivative as claimed in any one of claims 22 to 27 selected from

$$(A)\text{-}X_n\text{-}Y \qquad (II)$$

$$(A)\text{-}X_n\text{-}L\text{-}Y \qquad (III)$$

$$((A)\text{-}X_n)_m\text{-}L\text{-}Y \qquad (IV)$$

$$(A)-X_n-L-Y-L-X_r-(A) \qquad (V)$$

wherein A, X, L, n and r are as hereinbefore defined and $m \geq 2$.

29. An angiotensin derivative as claimed in any one of claims 22 to 28 selected from

N-acetyl-Cys-(A)

Tyr-(A)

N-acetyl-Cys-Gly-(A)

Cys - (A)

where A is angiotensin I

30. An angiotensin derivative as claimed in any one of claims 22 to 29 which elicits a cross-reactive immune response with angiotensin I, angiotensin II, and/or angiotensinogen molecules.

31. An angiotensin derivative as claimed in any one of claims 22 to 30 conjugated to a carrier.

32. An angiotensin derivative as claimed in claim 31 wherein said carrier is a polypeptide.

33. An angiotensin derivative as claimed in claim 32 wherein the carrier is selected from the purified protein derivative of tuberculin, tetanus toxoid, diphtheria toxoid, keyhole limpet haemocyanin or derivatives thereof.

34. A method of combatting conditions associated with activation of the renin-angiotensin system comprising

administering an angiotensin derivative as defined in any one of claims 1-17.

35. A nucleic acid molecule coding for a linear angiotensin peptide derivative as claimed in any one of claims 1-17, and nucleic acid molecules with sequences complementary thereto.

36. An expression vector comprising a nucleic acid molecule as claimed in claim 35.

37. A host organism transformed with the vector of claim 36.

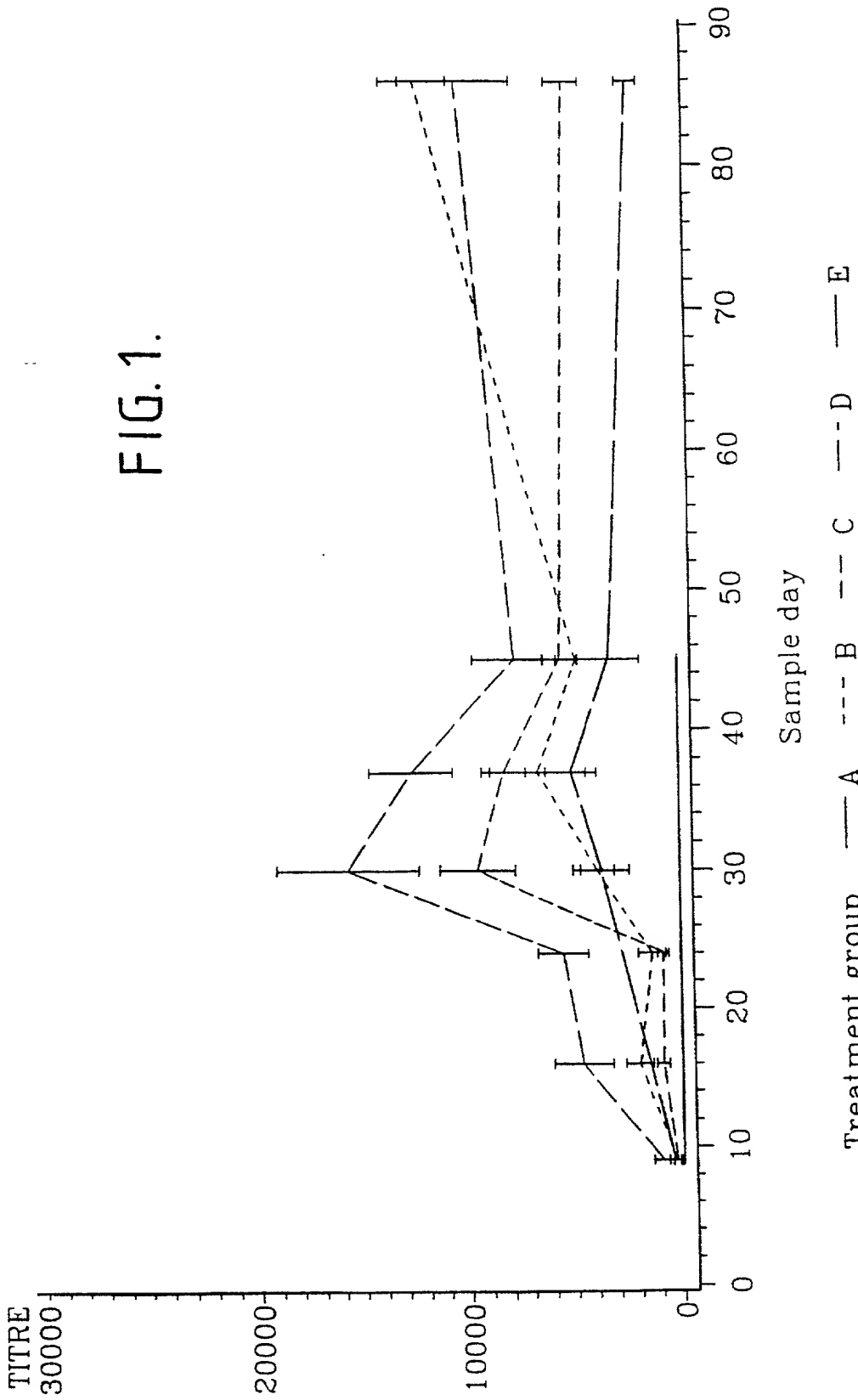
38. A method of combatting conditions associated with the renin-angiotensin system comprising administering a nucleic acid molecule coding for a linear angiotensin peptide derivative as claimed in any one of claims 1-16 or an expression vector comprising a nucleic acid molecule coding for an angiotensin peptide derivative.

39. A polypeptide immunogen capable when conjugated to a carrier of inducing antibodies in an immunised subject that recognise epitopes of angiotensin I, angiotensin II and/or angiotensinogen.

ABSTRACT

An angiotensin derivative comprising at least one angiotensin peptide moiety coupled to a peptide carrier-binding moiety which can be used for therapy and prophylaxis of conditions associated with the renin activated angiotensin system.

FIG. 1.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0
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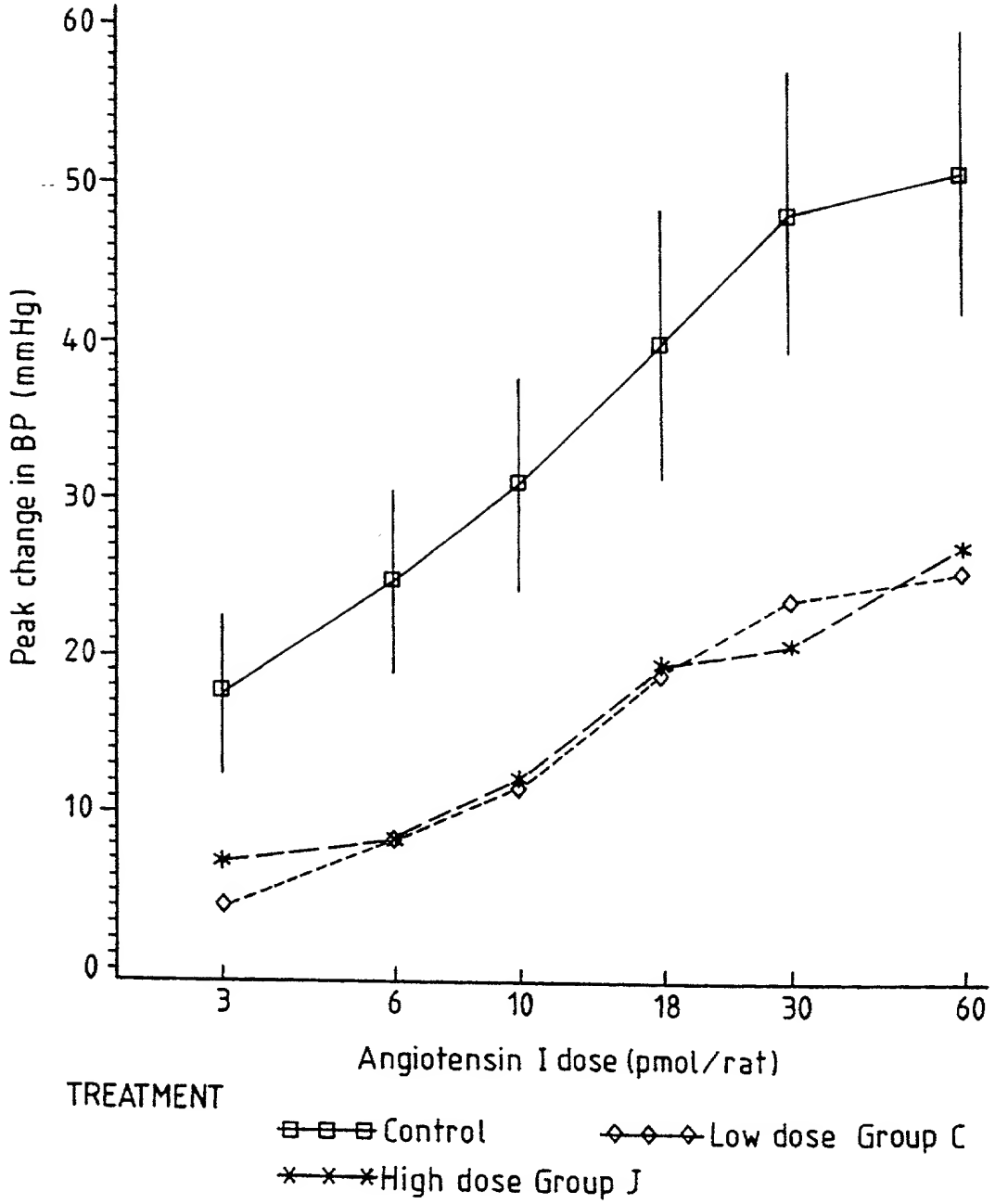
20
30
40
50
60
70
80
90
Sample day
Treatment group
A
B
C
D
E

FIG. 2.


60
50
40
30
20
10
0
Peak change in BP (mmHg)
3
6
10
18
30
60
Angiotensin I dose (pmol/rat)
TREATMENT
Control
Low dose Group C
High dose Group J

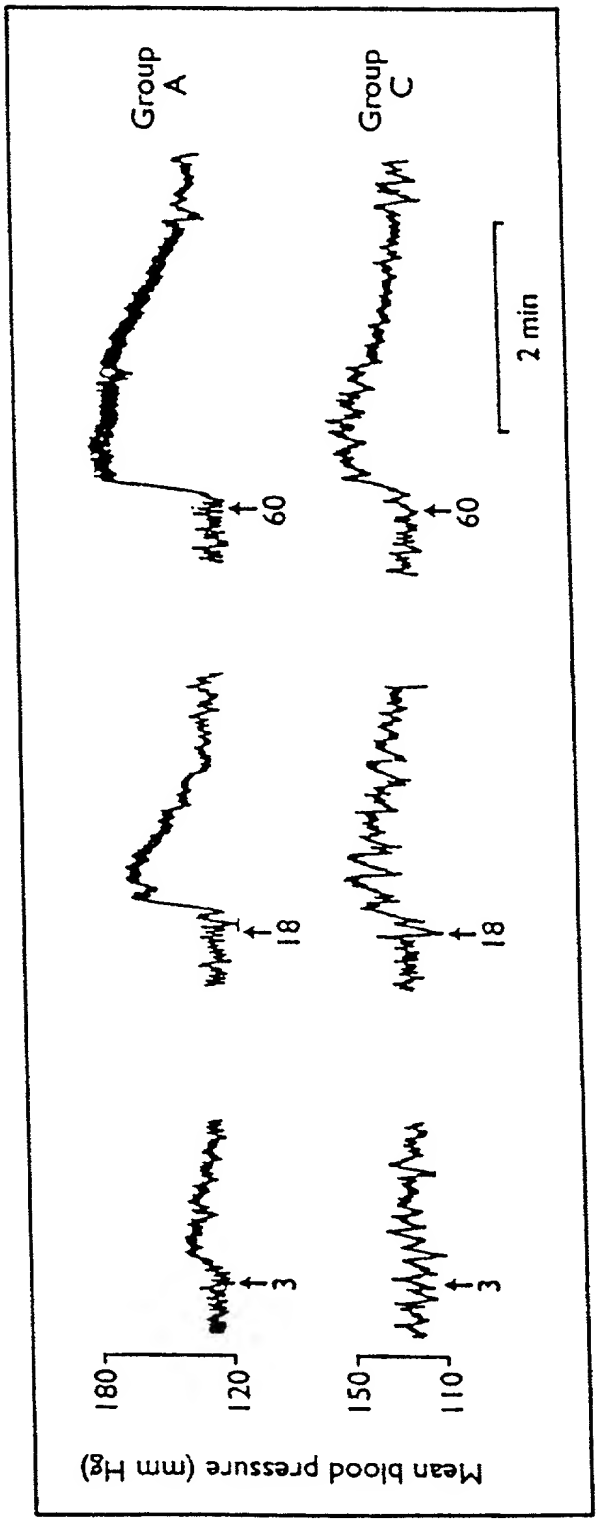
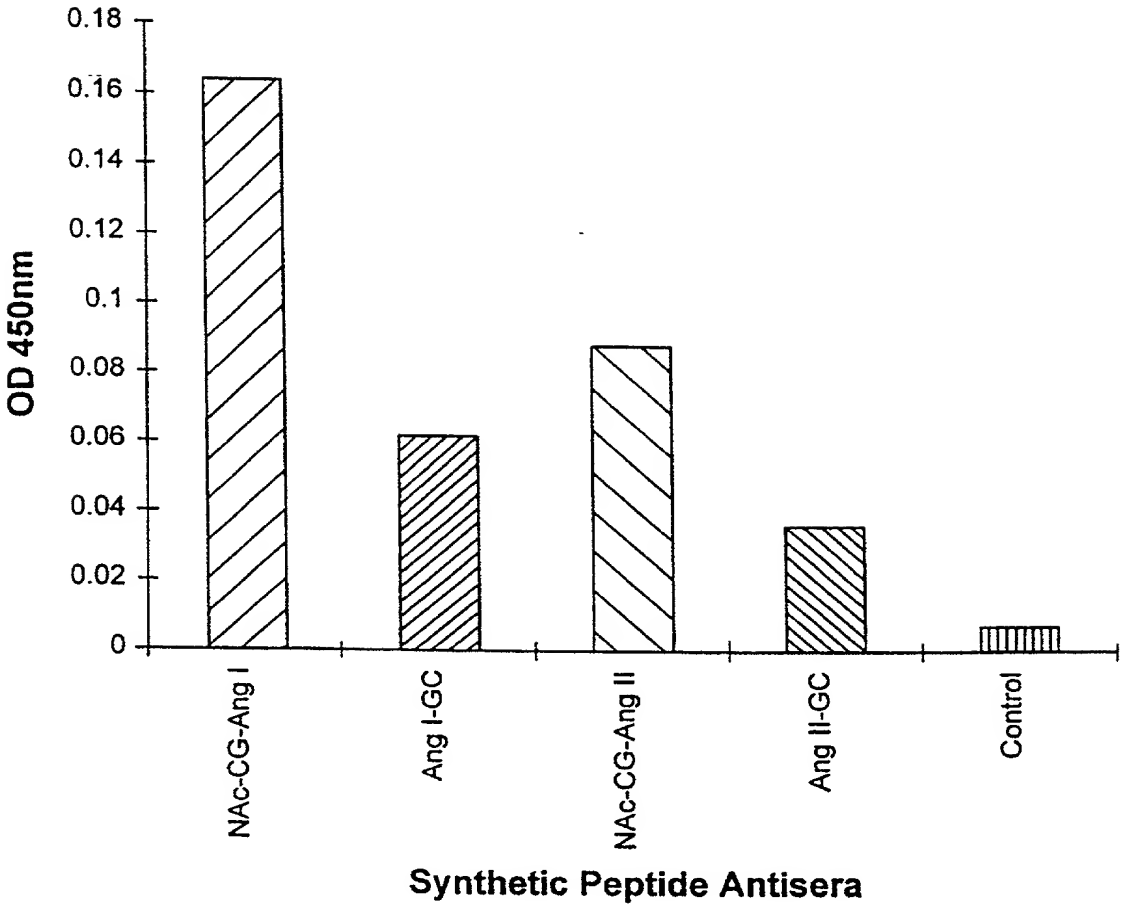

Group A
Group C
Mean blood pressure (mm Hg)
180
120
150
110
3
18
60
2 min

FIG. 3.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0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
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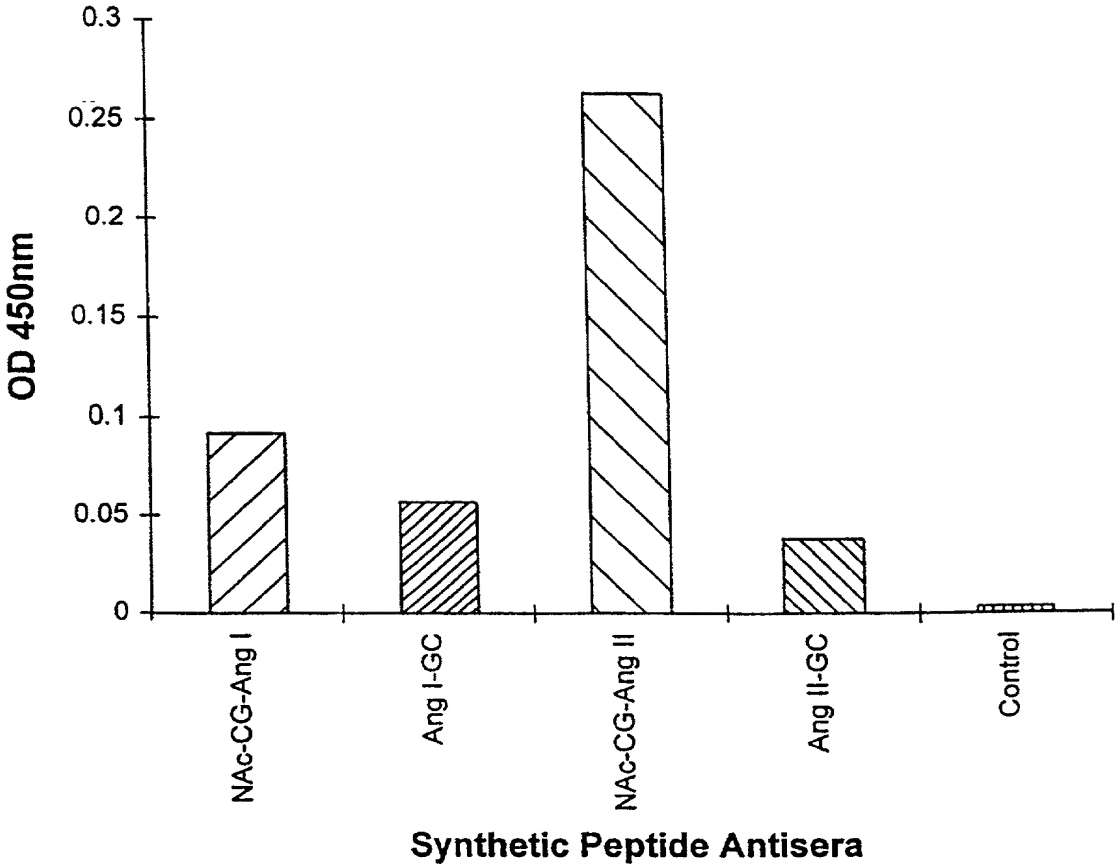
NAc-CG-Ang I
Ang I-GC
NAc-CG-Ang II
Ang II-GC
Control
Synthetic Peptide Antisera

FIG. 4.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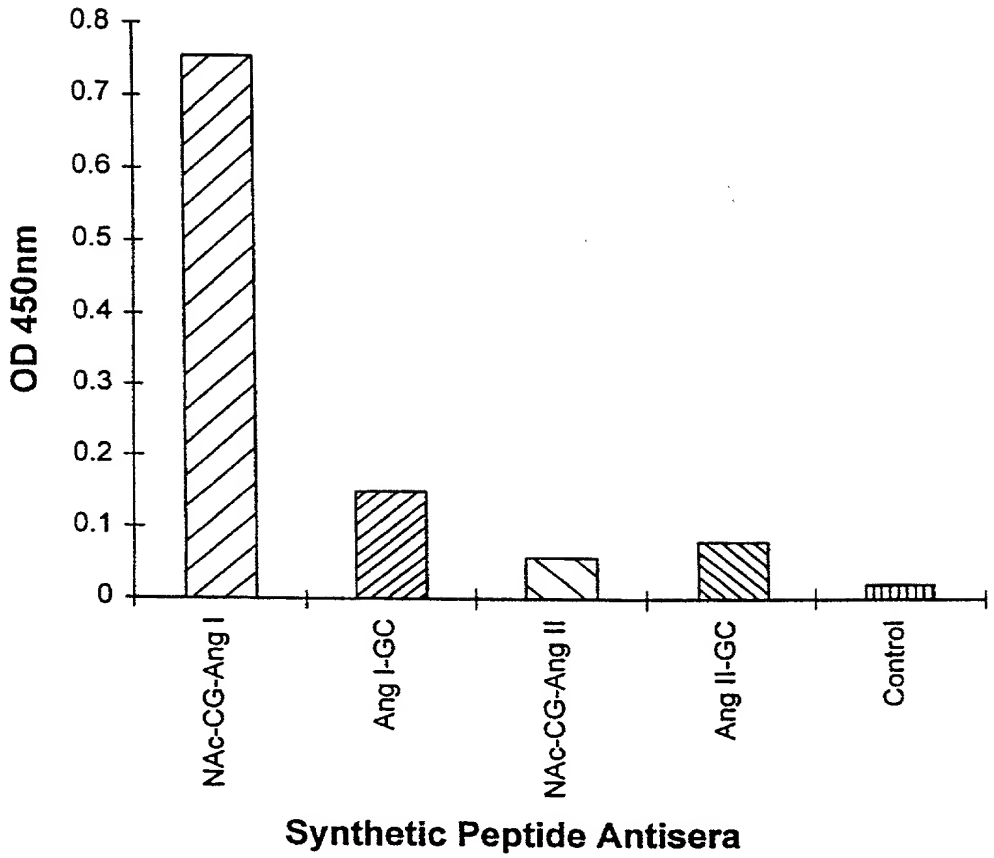
0.25
0.2
0.15
0.1
0.05
0
OD 450nm
NAc-CG-Ang I
Ang I-GC
NAc-CG-Ang II
Ang II-GC
Control
Synthetic Peptide Antisera

FIG. 5.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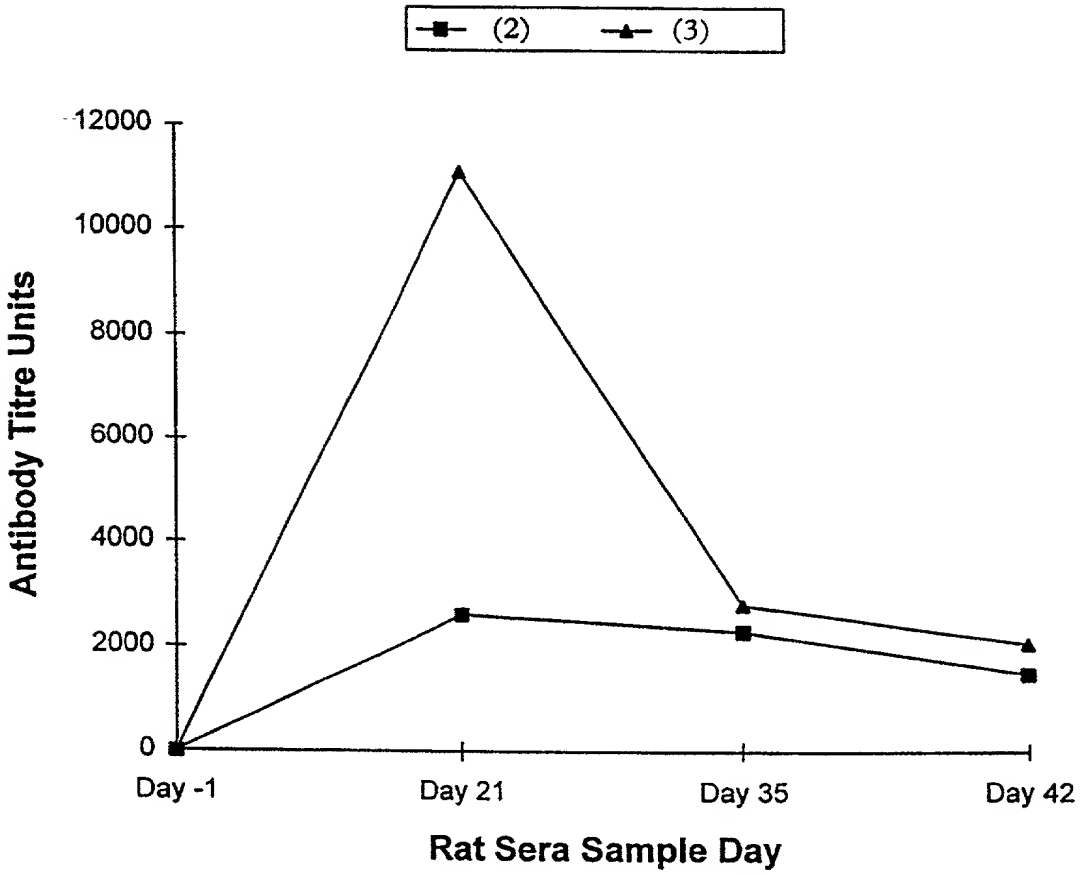
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 450nm
NAc-CG-Ang I
Ang I-GC
NAc-CG-Ang II
Ang II-GC
Control
Synthetic Peptide Antisera

FIG. 6.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(3)
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6000
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Antibody Titre Units
Day -1
Day 21
Day 35
Day 42
Rat Sera Sample Day

FIG. 7.

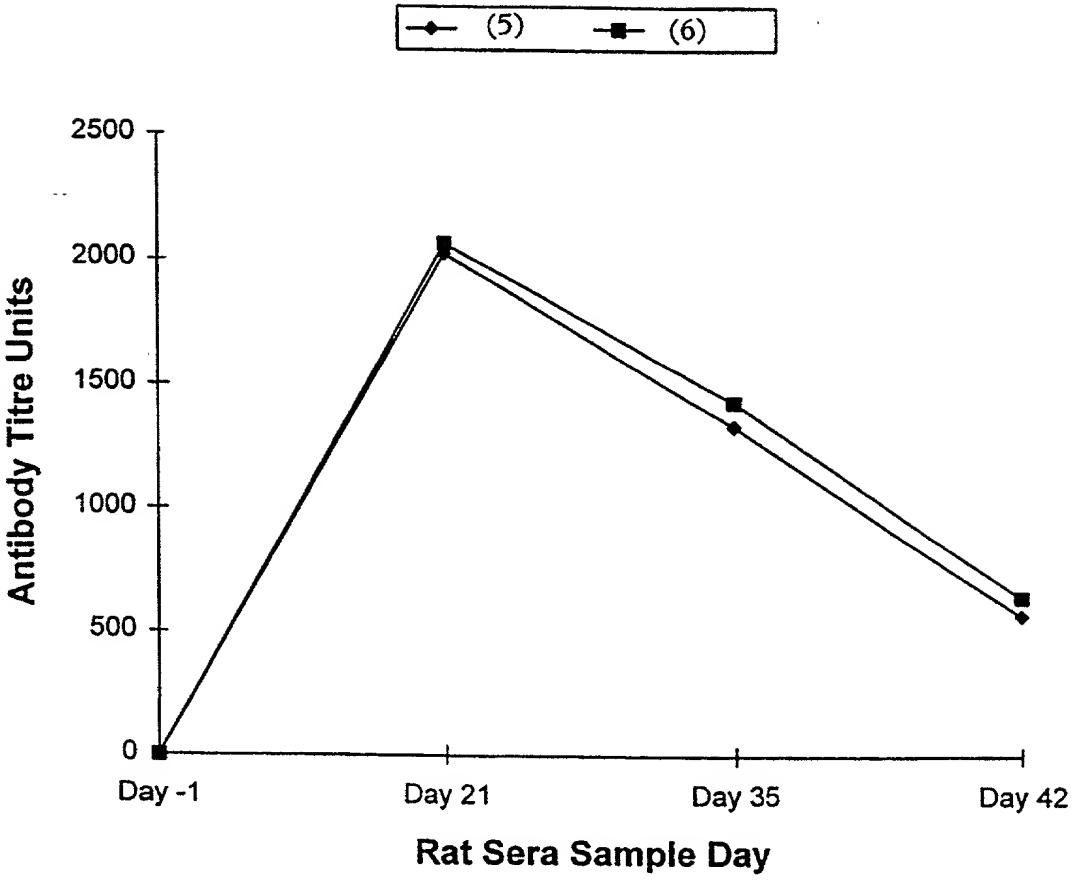
(5)
(6)
2500
2000
1500
1000
500
0
Antibody Titre Units
Day -1
Day 21
Day 35
Day 42
Rat Sera Sample Day

FIG. 8.

Attorney Docket No. 41565/192844

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION
(Foreign Agent Involved)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANGIOTENSIN DERIVATIVES

the specification of which is filed herewith as a continuation of the United States designation of PCT International Application Number PCT/GB98/01833 filed on June 23, 1998, and amended on May 26, 1999 and September 23, 1999.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed	
GB 9713361.5	Great Britain	June 24, 1997	[X] Yes	[ ] No
(Number)	(Country)	(Day/Month/Year Filed)		
GB 9808696.0	Great Britain	April 24, 1998	[X] Yes	[ ] No
(Number)	(Country)	(Day/Month/Year Filed)		

I hereby claim the benefits under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

_____ (Number) _____ (Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed

in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C., § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

PCT/GB98/01833	June 23, 1998	pending
(Appln. Serial No.)	(Filing Date)	(Status -- patented/pending/aban.)

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from my European representatives, Frank B. Dehn & Co., as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

I hereby appoint the practitioners associated with the firm of Alston & Bird, LLP to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to:

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North Building, 11th Floor
Washington, D.C. 20004

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Karen Lee Orzechowski Registration No. 31,621	Karen Lee Orzechowski Registration No. 31,621 Tel Washington Office 202-756-3371 Fax Washington Office 202-756-3333

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declaration and Power of Attorney for Patent Application
Attorney Docket No. 41565/192844
Page 3 of 4

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Attorney Docket No. 41565/192844

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